

On page 2, please insert the following heading after the second full paragraph that starts on page 1, line 16 and bridges page 2 and ends on line 29 and before the first full paragraph on page 2 that starts on line 30.

E5

--SUMMARY OF THE INVENTION--

On page 6, please delete and replace the current version of the first full paragraph starting at line 4 with the following replacement paragraph. Pursuant to 37 CFR § 1.121, the following is a clean version of the replacement paragraph. A marked-up version of the replacement paragraph is attached on a separate sheet.

E6  
More in particular the serine in position 2 or 3 is substituted with a cysteine and the cysteine is chemically modified with polyethylene glycol having a molecular weight of 5, 10 or 20 kDa. Preferred embodiments of these derivatives are SY161(S3C-MP5), SY161(S3C-P10), Sy161(S3C-P20), SY19(S3C-MP5), SY19(S3C-SP5), SY19(S2C-SP5, S3C-SP5), SY19(S3C-P20), SY19(S3C-P10) all as defined in table 20.

On page 6, please delete and replace the current version of the second full paragraph starting at line 12 with the following replacement paragraph. Pursuant to 37 CFR § 1.121, the following is a clean version of the replacement paragraph. A marked-up version of the replacement paragraph is attached on a separate sheet.

The presence of cysteines allows the formation of dimers of two staphylokinase derivatives of the invention.

On page 8 please insert the following heading before the paragraph starting at line 1 that begins with "**Fig 1**".

E7

--BRIEF DESCRIPTION OF THE DRAWINGS--

On page 8, please delete and replace the current version of the first full paragraph starting at line 1 with the following replacement paragraph. Pursuant to 37 CFR § 1.121, the

following is a clean version of the replacement paragraph. A marked-up version of the replacement paragraph is attached on a separate sheet.

**Fig 1.** Protein sequence of wild-type staphylokinase, SakSTAR. Numbering starts with the NH<sub>2</sub>-terminal amino acid of mature full length staphylokinase (SEQ ID NO: 1).

On page 8, please delete and replace the current version of the third full paragraph starting at line 13 with the following replacement paragraph. Pursuant to 37 CFR § 1.121, the following is a clean version of the replacement paragraph. A marked-up version of the replacement paragraph is attached on a separate sheet.

**Fig 3.** Protein sequence of wild-type staphylokinase, SakSTAR (SEQ ID NO: 1) with indicated amino acid substitutions.

squares: single amino acid substitutions;

circles: combined (2 to 3) amino acid to Ala substitutions.

On page 8, please insert the following heading after the fifth full paragraph starting at line 24 and ending on line 32 and before the heading "**EXAMPLES**" that starts on line 34.

--DESCRIPTION OF THE PREFERRED EMBODIMENTS--

On pages 12 and 13, please delete and replace the current version of the first full paragraph starting at page 12, line 25 and bridging page 14 and ending at line 25 with the following replacement paragraph. Pursuant to 37 CFR § 1.121, the following is a clean version of the replacement paragraph. A marked-up version of the replacement paragraph is attached on a separate sheet.

The plasmids encoding SakSTAR(K35A,E38A,K74A,E75A), SakSTAR(E38A,E75A,R77A), SakSTAR(E38A,E75A), SakSTAR(K35A,E75A,R77A), SakSTAR(K35A,E75A), SakSTAR(E80A), SakSTAR(D82A), SakSTAR(E75A,D82A),

E11  
SakSTAR(K74A) and SakSTAR(E75A) were constructed by the spliced overlap extension polymerase chain reaction (SOE-PCR) (24), using Vent DNA polymerase (New England Biolabs, Leusden, The Netherlands), and available or generated sakSTAR variants as template. Two fragments were amplified by PCR, the first one starting from the 5' end of the staphylokinase gene with primer 5'-CAGGAAACAGAATTCAGGAG-3' (SEQ ID NO: 2) to the region to be mutagenized (forward primer), the second one from the same region (backward primer) to the 3' end of the staphylokinase gene with primer 5'-CAAAACAGCCAAGCTTCATTCATTCAGC-3' (SEQ ID NO: 3). The forward and backward primers shared an overlap of around 24 bp (primers not shown). The two purified fragments were then assembled together in a new primerless PCR using Taq polymerase (Boehringer Mannheim). After 7 cycles (1 min at 94°C, 1 min at 70°C), the extended product was reamplified by adding the 5' and 3' end primers (see above) to the PCR reaction and by cycling 25 times (1 min at 94°C, 1 min 55°C, 1 min at 72°C). The final product was purified, digested with EcoRI and HindIII and cloned into the corresponding sites of pMEX602sakB. The plasmid encoding SakSTAR(E38A,K74A,E75A,R77A) was assembled by digestion of pMEX602sakB and pMEX.SakSTAR(K35A,E38A,K74A,E75A,R77A) with BpmI which cuts between the codons for K35 and E38 of SakSTAR, and ligation of the required fragments. The plasmid encoding SakSTAR(K35A,K74A,E75A,R77A) was constructed by digestion of pMEX.SakSTAR(K35A,E38A,K74A,E75A,R77A) and pMEX.SakSTAR(K74A,E75A,R77A) with BpmI and religation of the required fragments. The plasmids encoding SakSTAR(K35A,E38A,E75A,R77A) and SakSTAR(K35A,E38A,K74A,R77A) were constructed by two PCR using pMEX.SakSTAR(K35A,E38A,K74A,E75A,R77A) as template, followed by restriction ligation and recloning into pMEX602sakB.

On pages 23 and 24, please delete and-replace the current version of the second full paragraph starting at page 23, line 37 and bridging page 24 and ending at line 30 with the

following replacement paragraph. Pursuant to 37 CFR § 1.121, the following is a clean version of the replacement paragraph. A marked-up version of the replacement paragraph is attached on a separate sheet.

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The variants SakSTAR(Y17A,F18A), SakSTAR(F104A), SakSTAR(F111A), SakSTAR(Y9A), SakSTAR(Y91A), SakSTAR(Y92A), SakSTAR(I87A), SakSTAR(I106A) and SakSTAR(I120A) were constructed with the Chameleon Double-Stranded Site-Directed Mutagenesis kit from Stratagene (La Jolla, USA), using the pMEX.SakSTAR vector as template, and following instructions of the supplier. The mutagenic oligonucleotides (not shown) were used in combination with the selection-primer LY34 5' CAAAACAGCCGAGCTTCATTCATTCAGC (SEQ ID NO: 4), which destroys the unique HindIII site located 3' to the staphylokinase encoding gene in pMEX.SakSTAR and allows to counter-select the non-mutant progeny by HindIII digestion. The deletion of the HindIII site was in most cases correlated with the presence of the desired mutation introduced by the mutagenic oligonucleotide. The variant SakSTAR(I133A), was constructed by performing a polymerase chain reaction on the pMEX.SakSTAR plasmid using the primer 818A located at the 5' end of the sakSTAR gene (5' CAGGAAACAGAATTCAGGAG) (SEQ ID NO: 2) and the mutagenic primer LY58 (5' TTCAGCATGCTGCAGTTATTTCTTTCTGCAACAACCTTGG) (SEQ ID NO: 6). The amplified product (30 cycles: 30 sec at 94°C, 30 sec at 50°C, 30 sec at 72°C) was purified, digested with EcoRI and PstI, and ligated into the corresponding sites of pMEXSakSTAR. The variants SakSTAR(I128A), SakSTAR(L127A) and SakSTAR(N126V) were constructed by performing a polymerase chain reaction using the primer 818A located at the 5' end of the SakSTAR gene and mutagenic primers (not shown). The amplified product (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C) was purified, digested with EcoRI and StyI, and ligated into the corresponding sites of pMEXSakSTAR.

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On page 25, please delete and replace the current version of the first full paragraph starting at line 5 with the following replacement paragraph. Pursuant to 37 CFR § 1.121, the following is a clean version of the replacement paragraph. A marked-up version of the replacement paragraph is attached on a separate sheet.

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E13 The plasmids encoding all the other variants listed in Table 3 were constructed by direct PCR or by the spliced overlap extension polymerase chain reaction (SOE-PCR) (24) using pMEX.SakSTAR or available plasmids encoding SakSTAR variants as template. Two fragments were amplified by PCR (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C, the first one starting from the 5' end (primer 818A) of the staphylokinase gene to the region to be mutagenized (forward primer), the second one from this same region (backward primer) to the 3' end of the gene with primer 818D (5' CAAACAGCCAAGCTTCATTCATTCAGC) (SEQ ID NO: 5). The forward and backward primers shared an overlap of around 24 bp (primers not shown). The two purified fragments were then assembled together in a second PCR reaction with the external primers 818A and 818D (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C). The amplified product from this final reaction was purified, digested with EcoRI and HindIII and ligated into the corresponding site of pMEX.SakSTAR. For each construction, the sequence of the variant was confirmed by sequencing the entire SakSTAR coding region.

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On pages 29 and 30, please delete and replace the current version of the second full paragraph starting at page 29, line 18 and bridging page 30 and ending at line 6 with the following replacement paragraph. Pursuant to 37 CFR § 1.121, the following is a clean version of the replacement paragraph. A marked-up version of the replacement paragraph is attached on a separate sheet.

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E14 In an effort to maximize the activity/antigenicity ratio, these amino acids were substituted with other amino acids than Ala. As summarized in Table 5, substitution of K35 with Ala, Glu or Gln revealed that SakSTAR (K35A) had the most interesting properties, substitution

E14  
of Y73 with Phe, His, Lev, Ser or Trp did not rescue the marked reduction in specific activity, and K74 confirmed its key role in binding of antibodies from immunized patient plasma, the best specific activity/antigenicity ratios being obtained with SakSTAR (K74Q) and SakSTAR (K74R). SakSTAR (E80A,D82A) was preferred over the single residue variants SakSTAR (E80A) or SakSTAR (D82A) because of its somewhat lower reactivity with immunized patient plasma. SakSTAR (N95A) could not be further improved by substitution of N95 with Glu, Gly, Lys or Arg and it was unable to confer its increased specific activity to variants containing K74A or K135R. Finally SakSTAR (K130A) was outperformed in terms of specific activity by SakSTAR (K130T) and SakSTAR (V132A) by SakSTAR (V132R).

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On page 30, please delete and replace the current version of the second full paragraph starting at line 14 with the following replacement paragraph. Pursuant to 37 CFR § 1.121, the following is a clean version of the replacement paragraph. A marked-up version of the replacement paragraph is attached on a separate sheet.

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E15  
The SakSTAR (K130T,K135R) variant was taken as a template for additive mutagenesis because of its high specific activity with a moderate reduction of binding to antibodies of epitope cluster III and absorption of antibodies from immunized patient plasma (Table 6). Addition of G36R, K74R, or K74Q or both to the template did not markedly reduce the specific activity, reduced the reactivity with monoclonal antibodies against epitope cluster III (G36R substitution) and decreased the absorption of antibodies from immunized patient plasma (K74R or K74Q substitution). Combination of E65A or E65Q with K74Q in the SakSTAR (K130T,K135R) template reduced the absorption of antibodies from Pool 10 and Pool 40 to around 50 and 60 percent respectively, without markedly reducing the specific activity. Addition substitution of selected amino acids in the SakSTAR (E65Q,K74Q,K130T,K135R) template did not further reduce the antibody-absorption from Pool 10 or Pool 40. Surprisingly,

E15 the substitution of K136 with Ala and the addition of Lys in position 137 resulted in a marked increase in specific activity, as measured in the chromogenic substrate assay.

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On pages 43 and 44, please delete and replace the current version of the first full paragraph starting at page 43, line 19 and bridging page 44 and ending at line 5 with the following replacement paragraph. Pursuant to 37 CFR § 1.121, the following is a clean version of the replacement paragraph. A marked-up version of the replacement paragraph is attached on a separate sheet.

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E16 The variants SakSTAR (K102C) and SakSTAR (K109C), were constructed by the spliced overlap extension polymerase chain reaction (SOE-PCR) (24) using pMEX.SakSTAR encoding SakSTAR as template. Two fragments were amplified by PCR (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C), the first one starting from the 5' end (primer 818A) of the staphylokinase gene to the region to be mutagenized (forward primer), the second one from this same region (backward primer) to the 3' end of the gene with primer 818D (5' CAAACAGCCAAGCTTCATTCATTCAGC). The forward and backward primers shared an overlap of around 24 bp (for the construction of K102C: TAT GAT AAG AAT TGC AAA AAA GAA GAA (SEQ ID NO: 7) (backward) and TTC TTC TTT TTT GCA ATT CTT ATC ATA (SEQ ID NO: 8) (forward), for the construction of K109C: AAA AAG AAG AAA CGT GCT CTT TCC CTA (SEQ ID NO: 9) (backward) and TAG GGA AAG AGC ACG TTT CTT CTT TTT (SEQ ID NO: 10) (forward)). The two purified fragments were then assembled together in a second PCR reaction with the external primers 818A and 818D (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C). The amplified product from this final reaction was purified, digested with EcoRI and HindIII and ligated into the corresponding site of pMEX.SakSTAR. For each construction, the sequence of the variant was confirmed by sequencing the entire coding region.

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On pages 49 and 50, please delete and replace the current version of the second full paragraph starting on page 49, line 31 and bridging page 50 and ending at line 21 with the following replacement paragraph. Pursuant to 37 CFR § 1.121, the following is a clean version of the replacement paragraph. A marked-up version of the replacement paragraph is attached on a separate sheet.

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The variants SakSTAR(S3C,E65D,K74R,E80A,D82A,K130T,K135R), (SY19(S3C)), SakSTAR(S2C,S3C,E65D,K74R,E80A,D82A,K130T,K135R), (SY19(2SC,3SC)), SakSTAR(S3C,K35A,E65Q,K74Q,D82A,S84A,T90A,E99D,T101S,E108A,K109A,K130T, K135R,K136A,∇137K), (SY141(S3C)), SakSTAR(S2C,S3C,K35A,E65Q,K74Q,D82A, S84A, T90A,E99D,T101S,E108A,K109A,K130T,K135R,K136A,∇137K), (SY141(S2C,S3C)), SakSTAR(S3C,K35A,E65Q,K74Q,E80A,D82A,T90A,E99D,T101S,E108A,K109A,K130T, K135R), (SY160(S3C)) and SakSTAR(S3C,K35A,E65Q,K74R,E80A,D82A,T90A,E99D, T101S,E108A,K109A,K130T,K135R), (SY161(S3C)), were constructed by the spliced overlap extension polymerase chain reaction (SOE-PCR) (24) using pMEX.SakSTAR encoding SakSTAR as template, two fragments were amplified by PCR (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C), the first one starting from the 5' end (primer 818A) of the staphylokinase gene to the region to be mutagenized (forward primer), the second one from this same region (backward primer) to the 3' end of the gene with primer 818D (5' CAAACAGCCAAGCTTCATTCATTCAGC) (SEQ ID NO: 5). The forward and backward primers shared an overlap of around 24 bp. The two purified fragments were then assembled together in a second PCR reaction with the external primers 818A and 818D (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C). The amplified product from this final reaction was purified, digested with EcoRI and HindIII and ligated into the corresponding site of pMEX.SakSTAR. For each construction, the sequence of the variant was confirmed by sequencing the entire SakSTAR coding region.

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